

Perplexity vs Clarity in choosing the right molecular diagnostic techniques for SARS-COV2 detection in Indian setup

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ABSTRACT

After the havoc created by Spanish flu a century ago, the world is witnessing exactly a similar pandemic situation since the beginning of the year 2020. The unexplained respiratory illness with high morbidity & mortality which started in Wuhan, China and spread across the world was finally termed as COVID-19 disease caused by SARS-CoV-2 and later announced as pandemic by WHO. This novel virus SARS-CoV-2 is a new variant of SARS corona virus with high infectivity and mysterious pathophysiology. The major step towards containment of this pandemic is to scale up the testing for SARS-CoV-2 and thereby isolating and managing the patients at the earliest. Molecular amplification based methods such as Real time Polymerase chain reaction (RT-PCR), CBNAAT and TrueNAT are the most commonly used techniques for detection of SARS-CoV2. To utilize these diagnostic facilities optimally in the management of the suspected COVID 19 patients, it is of utmost importance for the healthcare providers to understand the intricacies related to these technologies. Thus, the technical details along with the pros & cons of these three amplification-based technologies for proper understanding of these diagnostic modalities for SARS COV-2 diagnosis are discussed herewith.

Keywords: CBNAAT and TrueNAT, clarity, diagnostic, molecular, perplexity, real-time Polymerase chain reaction (RT-PCR), SARS-CoV-2

Introduction

Considering the high rate of transmissibility along with its mysterious pathophysiology in the immune naïve population infected with SARS-CoV-2, it was of paramount importance to develop robust diagnostic tools for detection of this new variant of Corona Virus. Moreover, as the symptoms of COVID -19 can mimic other respiratory viral infections, thus the widespread testing capacity building for SARS-CoV-2 detection and diagnosis has been playing a pivotal role in identifying and isolating the

infected persons and thereby curbing the spread of the virus since the time COVID 19 has been declared as a Pandemic.

At present, we have various diagnostic modalities for detection SARS-CoV-2 targeting different components of the virus genomes as well detection of antibodies that are generated in response to COVID-19 infection. In India, the main amplification based laboratory diagnostic modalities for SARS-Cov-2 detection are Real Time Reverse Transcriptase Polymerase chain reaction (RT-PCR), Cartridge based nucleic acid amplification (CBNAAT) and Truenat technology. Having three different laboratory diagnostic platforms for detecting this novel virus, raised multiple queries among the clinicians and as well as in the community, regarding the intricacies and limitations of these methods.

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As it is a pandemic situation and number of cases are increasing rapidly, to curb the spread of the disease, detection and isolation of positive cases is of utmost importance. Keeping this in mind the general physicians who are attending these cases regularly in first instance, should know properly which test should be advised for the timely diagnosis. This article aims at analyzing the various boon or bane of each method to enrich the knowledge of general/primary care physicians regarding various testing methods so that they can apply this knowledge to advise the patient the best investigation for the timely diagnosis and necessary intervention. Moreover, this article will throw in depth light into the intricacies of these newer diagnostic tools for this new virus causing the pandemic.

SARS-CoV-2: Virology and target genes for diagnosis

Corona viruses are positive sense enveloped single-stranded RNA viruses with diameter size ranging from 80–220 nm. Under electron microscope, the envelop of the virus bears a crown-like, 20-nm in length spikes similar to corona of the sun, hence it is named as coronavirus. Coronaviruses are belongs to the family *Coronaviridae* subfamily *Coronavirinae* and order *Nidovirales*. This *Coronavirinae* subfamily is divided into 4 genera – *alpha coronavirus*, *beta coronavirus*, *gamma coronavirus*, and *delta coronavirus*. Till date, six coronaviruses are known to cause human diseases and among these two human coronaviruses are SARS-CoV and MERS-CoV are known to cause epidemic.^[1] Hence on finding the first sequence data of this novel human virus was placed in the *Sarbecovirus* subgenus of *Coronaviridae*, which was the same subgenus as the SARS virus which caused global outbreak in the year 2002-2003 with more than 8000 cases globally. On whole genomic sequencing of SARS COV-2 it was observed that this novel beta coronavirus differs from SARS-CoV.1 The virus spread widely within the Hubei province by mid-January of 2020 and by early March 2020 it spread to the other parts of the globe and henceforth WHO declared SARS COV-2 as pandemic.^[2]

SARS-COV-2 is a RNA virus with a positive strand, having a lipid bilayer envelop with four *structural proteins (S) envelope (E), nucleocapsid (N) and membrane (M) proteins*. There is an accessory protein which encodes 1/3rd of genome while the other 2/3rd genome is encoded by *ORF1a/b* polyproteins (which forms viral replicas transcriptase complex). It expresses their replication and transcription complex, through *RNA-dependent RNA polymerase (RdRp)*, from a single, large open reading ORF1ab³ Thus, the most commonly used targets for SARS-CoV-2 detection by RT-PCR are *ORF1ab/RdRp, E, N, and S genes*^[3] RT-PCR technology relies on its ability to amplify a smaller amount of viral genetic material (if present) in a given sample and because of its sensitivity and specificity, it is considered to be the gold standard for identification of SARS-CoV-2 virus. Currently, upper respiratory tract samples (nasopharyngeal, oropharyngeal swabs) are mainly used for SARS –CoV-2 RT-PCR tests.^[4,5]

1. Real time Reverse transcriptase PCR for SARS-CoV-2:

This is a real-time reverse transcriptase polymerase chain reaction (rRT -PCR) test which can be run either in a singleplex

format (individual targets in individual wells) or multiplexed formats (multiple targets in a single well). Amplification set up with a *human RNase P (RNP)* in a clinical sample as a human specimen control which is used to ascertain the quality of sample collection. RNA isolated from nasopharyngeal and oropharyngeal swabs, is reversely transcribed to form a Complementary DNA (cDNA) strand and then it is amplified multiple times using thermocycler machine which provides stringent conditions for the amplification reactions to happen. The fluorescence signals emitted from the Taqman probes is captured by the CCD camera and amplification plot is generated in the exponential phase.

Conventional PCR is one of the most frequently used molecular technique for diagnosis of infectious disease. However, post amplification processing such agarose gel electrophoresis and less sensitivity of conventional PCR makes it an unsuitable approach for diagnosis of SARS-CoV2 as Covid-19 demands prompt diagnosis for better management and isolation of the patients. Real Time RT -PCR is a specialized version of PCR which can directly amplify the viral RNA from the clinical samples and obviates the need of post amplification end point analysis and the amplification which can be monitored in real time.^[6]

Principle of Real time PCR:

Real-time PCR uses the technique of analysing data through the PCR where it includes the combination of single step amplification and detection through fluorescence capture technique. It utilizes a different fluorescent dyes that directly correlates genomic product concentration which is amplified to fluorescence intensity.^[7] Here, the reactions are characterized by the time point, where the target amplification is detected first in the exponential phase. This value is usually known to as cycle threshold (C_t), which implies the time at which detectable fluorescence intensity is higher than the background fluorescence. To paraphrase, the higher the quantity of genetic material existing in the clinical sample, the earlier significant increase in fluorescent signal will generate, yielding a lower C_t .^[8,9] The Real Time-PCR run for molecular diagnosis is carried out along with known positive controls, no amplification control and no template control.^[10]

The RT-PCR amplification curve has four phases, such as a) baseline b) exponential c) linear and d) plateau. The baseline phase is the one where all the amplification plots are below the detection level. The exponential phase is described as the earliest detectable fluorescent signal where the amplification of genetic material is taking place in the exponential phase which is dependent on the concentration of the template in the sample. This phase is followed by the linear amplification plot where the amplification begins to taper off where curve resembles as a straight line and the amplification plot gradually declines when it reaches the plateau phase.

A. Methodology of Real time PCR: [Figure 1]

- **Sample collection:** The most commonly used samples for real time PCR are both nasopharyngeal swab and

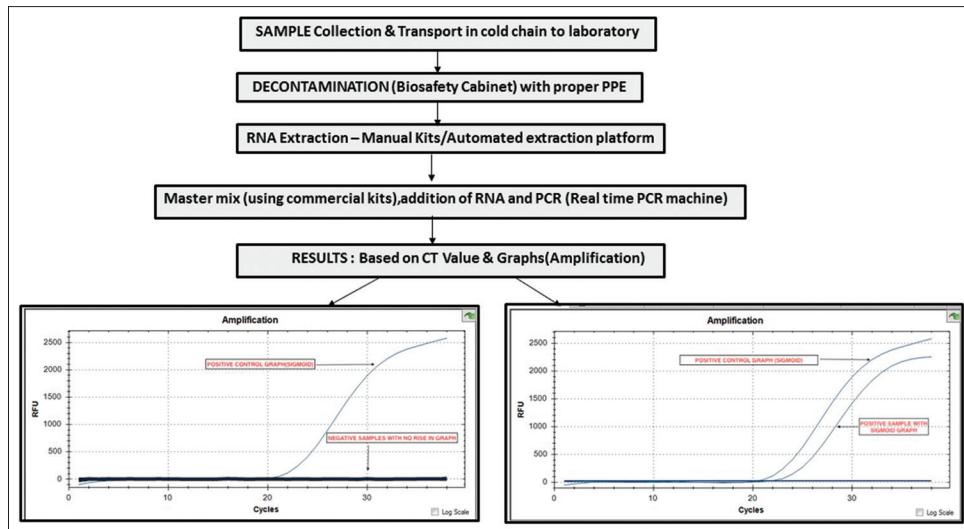


Figure 1: Flow chart showing stepwise procedure and interpretation of Real time PCR

oropharyngeal swab. Under special conditions like when patient is in intubation, Et secretion or Tracheal aspirate, BAL aspirate can also be collected. Many a times in particularly in post mortem cases nasal swabs can be tested. The samples are collected in Viral Transport Medium (VTM) and are transported to the laboratory maintaining the proper cold chain. As SARS-COV 2 being a RNA virus, it needs cold environment to be stable so that it can be detected in Real time PCR.^[11]

- **Decontamination:** It is the first step for processing of clinical samples in laboratory. As the sample is received in the lab, it is opened in a biosafety cabinet with proper PPE and then lysis buffer is added to inactivate the viral proteins and render the sample non-infectious. The average Time taken is 10-15 minutes per sample and it is performed in batches.
- **RNA Extraction:** The Samples post proper decontamination are processed for RNA extraction. RNA extraction is done by commercial kits either manually or in an automated platform. ICMR has approved 5 kits for manual extraction dated 1/4/2020.^[12] For manual extraction time taken varies according to number of samples. On an average it takes around 1-2 hours for extraction of 96 samples. However, in case of an Automated extractor system it varies according to load capacity of the machines which ranges of 24, 64 and 96 samples at a single time and taking a time from one hour to 2 hours to complete the entire extraction.

Mastermix preparation and Real time PCR:

There are various master mix kits approved by ICMR: In a communication by ICMR Dated 7/8/2020 it has approved 94 number of kits to be satisfactory for COVID PCR.^[13]

Master mix involves the mixing of various reagents in a PCR tubes which include: Reaction mix, RNase free water, Primer probe mix and One step RT PCR reagent (in different volumes based on the various kits literature). The template RNA which

has been extracted are added in this master mixture [Table 1]. This step takes approx. one hour for 96 samples.^[14-19]

The Entire mixture in a PCR tubes is then put inside a Real time PCR machine in which the mixture undergoes various PCR steps (Reverse transcription, activation, denaturation and annealing). The time taken for this varies according to kits available [Table 1]

The results are thereafter interpreted based on C_T value (Cycle threshold) and nature of the curve. The C_T value for interpretation of test to be positive varies according to the kits. [Table 1] However, the graph that is observed to declare a positive should be sigmoid.

As it was mentioned earlier that ICMR has approved quite a number of Real Time PCR Kits. In the Table 1 we are trying to compare the attributes of 5 different kits which are often used across various SARS-CoV-2 testing laboratories and are provided by ICMR.

Overall Procedure of Real Time RT PCR for SARS-CoV-2 and interpretation of results is depicted in Figure 1

Issue related to Interpretation of C_T value and Viral load:

It has been observed that C_T value is inversely proportion to the viral load theoretically. High viral load suggests increase infectiveness along with severity of the disease. However, this is a robust finding based on assumptions and limitations.

To clear the confusions related to C_t values as a guide for patient management, ICMR has released an advisory on the correlation of C_t values of real time RT-PCR test with COVID-19 disease severity. It has been observed that the C_t value varies according to the various kits used, the type of sample collected and sample collection procedure, transport procedure of the specimen etc., Moreover, an asymptomatic/mild symptomatic cases might

Table 1: Different commonly used kits approved by ICMR for Real time PCR (kit literature)^[13-19]

Manufacturer	NIV, Pune	True PCR	TaqPath™	BGI	Lab Gun
Type of assay	Single plex assay	Single plex assay	Multiplex assay	Duplex assay	Single plex assay
Target genes	Screening: E gene Confirmatory assay : ORF, RdRp RNase P (internal control)	Viral targets: E gene, N gene RNase P (internal control)	Viral targets: ORF1 Ab, cN gene, S Gene MS2 (internal control)	ORF1Ab Internal Control	Viral Targets: E gene, RdRP Internal Control
Cycling Condition:	Reverse transcription: 55°C for 30 min Taq inhibitor inactivation: 95°C for 3 min	cDNA synthesis: 50°C for 15 mins Activation : 95°C for 5 mins	Incubation : 25°C for 2 mins Reverse transcription: 53°C for 10 mins Activation : 95°C for 2 mins Denaturation: 95°C for 3 sec	Reverse transcription: 50°C for 20 min Initial denaturation: 95°C for 10 min	cDNA synthesis: 50°C for 30 mins Pre denaturation: 95°C for 15 mins Denaturation: 95°C for 15 sec (45 cycles)
Analysis & Interpretation	PCR amplification (45 cycles: 95°C for 15 sec 58°C for 30 sec* data analysis Total time : 90 mins approx Clinical samples : RNase P : Ct 35 cycle POSITIVE: E gene and either RdRp or ORF or both gene positive: POSITIVE with CT <35 RNase P: Indicates the presence of sufficient RNA from human RNase P gene indicating the specimen is of acceptable quality	PCR amplification (30 cycles: 95°C for 5 sec 60°C for 40 sec 72°C for 15 sec Total time : 90 mins (approx.) Clinical samples : RNase P : Ct 22+/-5 cycle E gene & N gene positive: POSITIVE Only N gene positive: POSITIVE Only E gene POSITIVE: Sabechevirus positive Cut off of CT value is 36 to label as Positive	Anneal/extension: 60°C for 30 sec (45 cycles) Total time: 85 mins Test specimen: Inconclusive results.Repeat test is advised: Only one SARS CoV2 Target positive: POSITIVE : Two or more SARS CoV2 Target positive: Internal control MS2 : Positive (test Valid) MS2 CT value <=32 Viral targets CT value <=37	PCR amplification (40 cycles: 95°C for 15 sec 60°C for 30 sec* data analysis Total time : 70 mins approx Test specimen: Positive - Ct value <38 and sigmoid curve Internal Control - Ct value <32 and sigmoid curve	Anneal/extension: 60°C for 1 min (45 cycles) Total time : 140 mins (approx.) Test specimen: POSITIVE: RdRP positive or both RdRP and E gene positive Note: CT value of <40 is taken as positive Internal control : Positive test valid
Run validity	Negative template control : should not exhibit fluorescence Positive Control :should have graph between 20 and 30 cycles				
Limit of Detection	Not mentioned	6 copies/ul with 95% of all	10 GCE/reaction	100 copies/ml (95% detection, throat swab)	100 copies/reaction (95% CI)

have same Ct value like that of a severe symptomatic case of COVID-19. This finding points to the conclusion that there is no direct correlation between the disease severity and the CT values. Rather the disease severity and patient outcome depends on various factors like immune status of the patient, presence of co morbid conditions etc., One thing worth noting here is that the RT PCR that are being performed presently are qualitative in nature and doesn't measure or quantify the viral load^[20]

Advantages of RT-PCR for SARS-CoV-2:

1. High precision with increased sensitivity and specificity.
2. It's a robust technique which is well acquainted by many medical staffs.
3. Lots of samples can be processed together in 96 well microtiter plates.
4. Due to presence of Positive and negative controls the results are validated in each PCR run minimizing the chances of false positive and false negative results
5. There is presence of human gene in most of the RT PCR kits as an extraction control which helps to determine whether the sample collected and RNA extracted out of it is adequate.

6. Different target genes of SARS-CoV-2 have been evaluated and validated in different kits. So there is an option for choosing the right kit as per the need.

Disadvantages of RT-PCR for SARS-CoV-2 :

1. Lots of technical expertise is required to perform and interpret the test
2. Reagents are to be transported and stored in controlled environment i.e., -20⁰ C.
3. At least Biosafety level 2 (BSL) type of laboratory is required to perform this test, so its not for basic laboratories. Laboratories need to be designed with proper workflow. If proper biosafety practices are not performed there shall be risk of occupational health hazards along with contamination of the samples which may interfere with the results giving rise to false positivity.
4. The sensitivity and specificity vary based on the kits. So, results might vary and depends on subjective error due to human interference
5. Time consuming as it requires multiple steps.
6. Quality control and calibration of instruments are important to get accurate & validated results.

7. High cost of consumables and various sophisticated equipment involved in Extraction and PCR process.

Apart from this the RT PCR for SARS-CoV-2 has following limitations:

1. Timeline of disease progression, type of sample and sample quality are other factors which contribute to diagnostic uncertainty
2. As long incubation time of the disease varies and in initial days of illness there is a low viral load it may produce a false negative result. In many cases reported false negative cases, patients did not carry enough viral load to be detected positive at the time of sampling.^[21]
3. Moreover, a negative result from a respiratory sample can only tell whether the virus is cleared from the respiratory tract but it's difficult to interpret whether it has been cleared from other body fluids or not.^[22]

B. Cartridge based Nucleic acid amplification technique (CBNAAT), for Detection of SARS-COV-2

CBNAAT (GeneXpert) is an in-vitro qualitative nested real-time polymerase chain reaction. This cartridge-based automated molecular diagnostic modality was initially endorsed by WHO for detection of Mycobacterium tuberculosis and rifampicin resistance for both pulmonary and extra pulmonary Tuberculosis in children within two hours^[23]

CBNAAT FOR SARS –COV 2:

Principle: This automated system is also based on the basic principle of rRT-PCR to detect SARS-COV2, but it integrates the various steps like the sample preparation, viral RNA extraction, amplification as well as detection of target sequences in a single cartridge.^[24,25] It uses single-time disposable cartridges that contains the RT-PCR reagents like primers, probes and internal control and perform the RT-PCR in GeneXpert Instrument systems. This system comprises of an instrument

that holds cartridges [Figure 2] computer with specific software for running tests and interpretation of graphs. The different modules of gene expert are available with 1, 2, 4 or 16 cartridge configuration.

Sample type: Upper respiratory specimens such as nasopharyngeal and oropharyngeal swabs are the preferred samples for CBNAAT just like RT PCR for SARS-COV-2

The components of the system are^[26]

- i. Primers specific for the COV genes for the RNA detection from SARS-COV-2 in upper respiratory samples.
- ii. Sample Processing Control (SPC): The SPC depicts for adequate sample processing and for the presence of potential inhibitor (s) in the RT-PCR reaction. The SPC also indicates that the reaction conditions like temperature, amplification time and reagents (primers, probes etc) for RT-PCR are functional.
- iii. The Probe Check Control: This verifies that the components to perform the reaction are present in the cartridge, checks PCR tube filling, reagent rehydration as well as confirms and monitors the dye stability and probe integrity

Method of Performing CBNAAT for SARS-CoV-2: (as explained in the Figure number 2)

1. The upper respiratory specimens are collected in a Viral transport media (VTM) containing either 3 mL of VTM or 3 mL of saline and transported to lab maintaining the proper cold chain.
2. In the laboratory, the specimen after receiving in the laboratory is mixed by rapidly inverting the collection tube 5-6 times and then transferred to the sample chamber of the Xpert Xpress SARS-CoV-2 cartridge.
3. The cartridge containing the sample is loaded into the GeneXpert Instrument systems that performs the entire rRT-PCR.

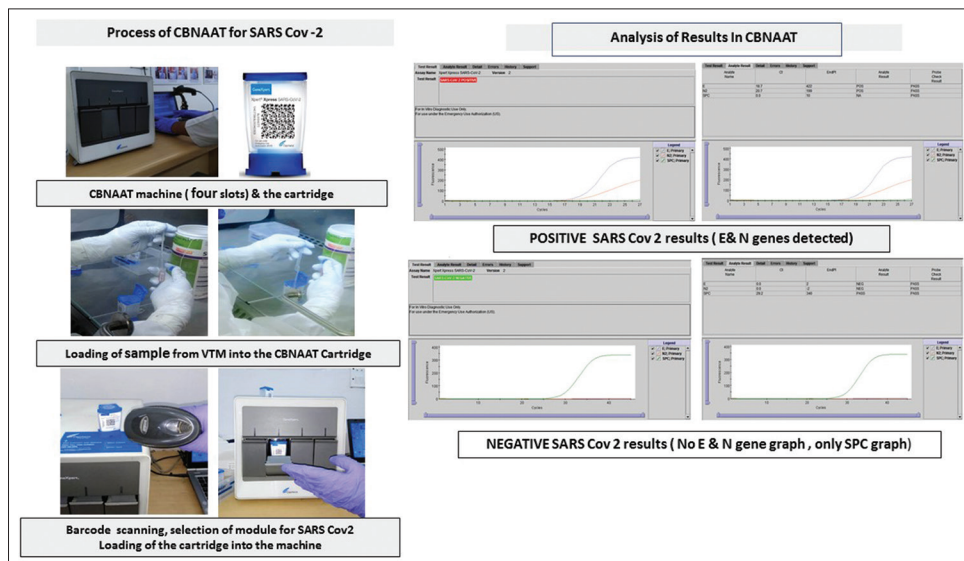


Figure 2: Workflow with analysis of results in CBNAAT platform for detection of SARS Cov-2

Target Genes:

- *E (Envelope) gene*: Screening gene for detection of **SARS-COV2**
 - *N2 (Nucleocapsid gene)*: Confirmatory gene for the the SARS-COV2 virus
- (Both these genes are detected simultaneously in single run only)**

Result interpretation for SARS-COV-2:

- **Positive for SARS-COV2:** If the Ct values for **both** *N2* and *E* or **only the N gene** are within the valid range irrespective of the SPC that can be positive or negative, the sample is considered positive.
The negative SPCs can be ignored as the target amplification has occurred.
- **Presumptive Positive for SARS-COV2:** If the SARS-COV-2 signals for **only the E nucleic acid target** irrespective of the SPC that can be positive or negative. The negative SPCs can be ignored as the target amplification has occurred.
In this case sample can be retested and if the same result is coming, the resample of the patient can be asked after 5-7 days of initial test.
- **Negative result for SARS-COV2:** SARS-COV-2 target *N2* and *E* gene are not detected.

Performance Characteristics:

According to manufacturer's instructions, the Positive percent (PPA) was, 97.8% (95% CI: 88.4% - 99.6%) and Negative percent agreement (NPA) was, 95.6% (95% CI: 85.2%-98.8%).⁽²⁷⁾ The limit of Detection (LOD) or analytical sensitivity of Live SARS-COV2 virus is 0.0200 PFU/ml. LOD is defined as the lowest concentration of live SARS-CoV-2 viral particles present in the samples that can be reproducibly distinguished from negative samples in more than equal to 95% of the time with 95% confidence and it is expressed in PFU/ml (plaque forming unit/ml)

Advantages of CBNAAT for SARS-CoV-2:

1. Cross-contamination between samples is minimized as the cartridges are self-contained.
2. This system has a quick turnaround time (approx. 1 hr 45 minutes) that includes the nucleic acid extraction time.
3. The screening and confirmatory genes are done in single run.
4. The confirmatory gene N, nucleocapsid gene is specific for SARS-COV-2
5. This platform has widespread availability even at district and primary health centre level as it has been widely used for diagnosis of Tuberculosis and other infectious diseases.
6. A very simple method with minimal hands -on technical time if the quantity of organisms

Limitations of CBNAAT for SARS-CoV-2:

- The proper temperature control as well as annual calibration of instrument is must.
- Uninterrupted power supply is required (with additional batteries or a generator can be attached).
- Performance evaluation and validation of CBNAAT results

for SARS-CoV-2 was mainly done on nasopharyngeal swabs and other nasal specimens such as nasal wash/aspirate. Validation yet to be performed on other upper respiratory samples such as oropharyngeal swab, nasal swab

- Factors such as improper and inadequate sample collection and transportation may affect the quality of the results giving rise to false negative results in such samples.
- Ongoing mutations within the target sequence of SARS- CoV-2 genes such as *S* and *N2*, can alter the configuration of binding sites for primer and/or probe leading to the failure of the amplification process.

C. Truenat testing for SARS-COV-2 detection^[27]:

Truenat technology for detection of SARS-COV-2 is a Make in India technology which was already in use in various RTNCP centres across India in the diagnosis of Pulmonary tuberculosis. Hence, in the present COVID-19 pandemic, the company has introduced a new platform and chips in the existing Truenat machines for detection of SARS-COV-2, which is an important addition in the ongoing quest for robust diagnostic methods for COVID19.

Principle:

Truenat technology for detection of SARS-COV-2 is again a real time reverse transcriptase PCR which uses a chip based platform. This diagnostic platform uses two different chips for quantitative detection of beta coronavirus (sarbeco virus) and SARS-CoV-2 RNA respectively.

Target Genes:

- *E (Envelope) gene*: Screening gene for detection of beta coronavirus (Truenat beta Cov chip)
- *Rdrp (RNA dependent RNA polymerase) gene*: Confirmatory gene for final detection of SARS-CoV-2 (Truenat SARS-CoV-2 chip)

Sensitivity & Specificity of the kit has been mentioned as 100% in company product brochure and they have also claimed that there is no cross reactivity to any other respiratory pathogen.

Limit of detection (LOD):

The LOD of the two chips targeting *E* & *Rdrp* gene in Truenat platform is more or less same which is estimated to be around 486 genome copies/ml for beta Cov Chip and 407 genome copies/ml for SARS-CoV-2 chip. The results were compared after a high titre sample was serially diluted and both Truenat Beta Cov and SARS-CoV-2 RT PCR was run side by side. The results of Truenat were found to be promising.

Flow of Work For Truenat :

NPS/OPS samples are received in a specialized vial containing lysis buffer made for Truenat (Point to note: sample sent for Truenat in this special vial cannot be tested for conventional RT PCR or CBNAAT). The lysis buffer inactivates the virus (if it is present in the sample) making it non-infectious. Therefore

biosafety requirements are minimal while performing Truenat testing. Steps involved in Truenat as described in the Figure 3.

Time taken for completion of the test:

RNA extraction time :18 minutes.

Truenat beta cov (E gene detection) and Truenat SARS-CoV-2 testing time: 42 min.

So the confirmed negative result can be obtained in around one hour (extraction time plus detection of E gene) and for screening test positive result another 42 min is required to get the confirmed positive/negative results for SARS-CoV-2.

Result Interpretation:

- The progress of the test can be visualized on the screen of the analyzer by observing the two amplification curves
- **POSITIVE Result:** It is indicated by the rise of Target graphs (E/RdRp) as well as the internal positive control (IPC) graphs in an exponential fashion and fluorescence crossing the threshold value. Rise of IPC curve is essential for the validity of the test result
- **NEGATIVE result:** There is no rise of the target graphs and they remain horizontal throughout the amplification process. Only the IPC graph shows an exponential rise indicating that the test run was valid
- **RESULT INVALID:** There is no rise of IPC curve and it remains horizontal at the end of the test. (Invalid samples should be repeated with fresh specimen from the sample

preparation stage)

- The positive results are also accompanied by icons like “HIGH”, “MEDIUM”, “LOW” or “VERY LOW” corresponding to the viral load of each sample as claimed by the kit instruction.

Guidelines issued from ICMR regarding performing and reporting of TRUENAT tests for SARS-CoV-2^[28]

According to the ICMR advisory, all samples of suspected SARS-CoV-2 infection should be tested for E gene assay (Truenat Beta Cov Chip) first while using Truenat platform. The samples which are tested negative for E gene are considered as True Negative or Confirmed negative.

All the samples that are tested positive by E gene assay (Truenat Beta Cov chip) are further tested for of *RdRp* gene (Truenat SARS-COV-2 chip) which is a confirmatory gene for SARS-CoV-2. The samples that are tested positive by this final assay for *RdRp* are considered as True positive or Confirmed positive and there is no need of further RT PCR testing for these positive samples. Recently, Multiplex Truenat Assay has also been developed and it has been approved by ICMR. This assay uses E gene as screening and Orf1a as confirmatory gene for COVID 19 detection in a multiplex format.^[29]

Advantages of Truenat testing:

1. Minimal Biosafety requirements as the sample is in a specialized medium where due to lysis buffer the virus become inactivated/noninfectious. That is why no special infrastructure is required and a basic healthcare facility can

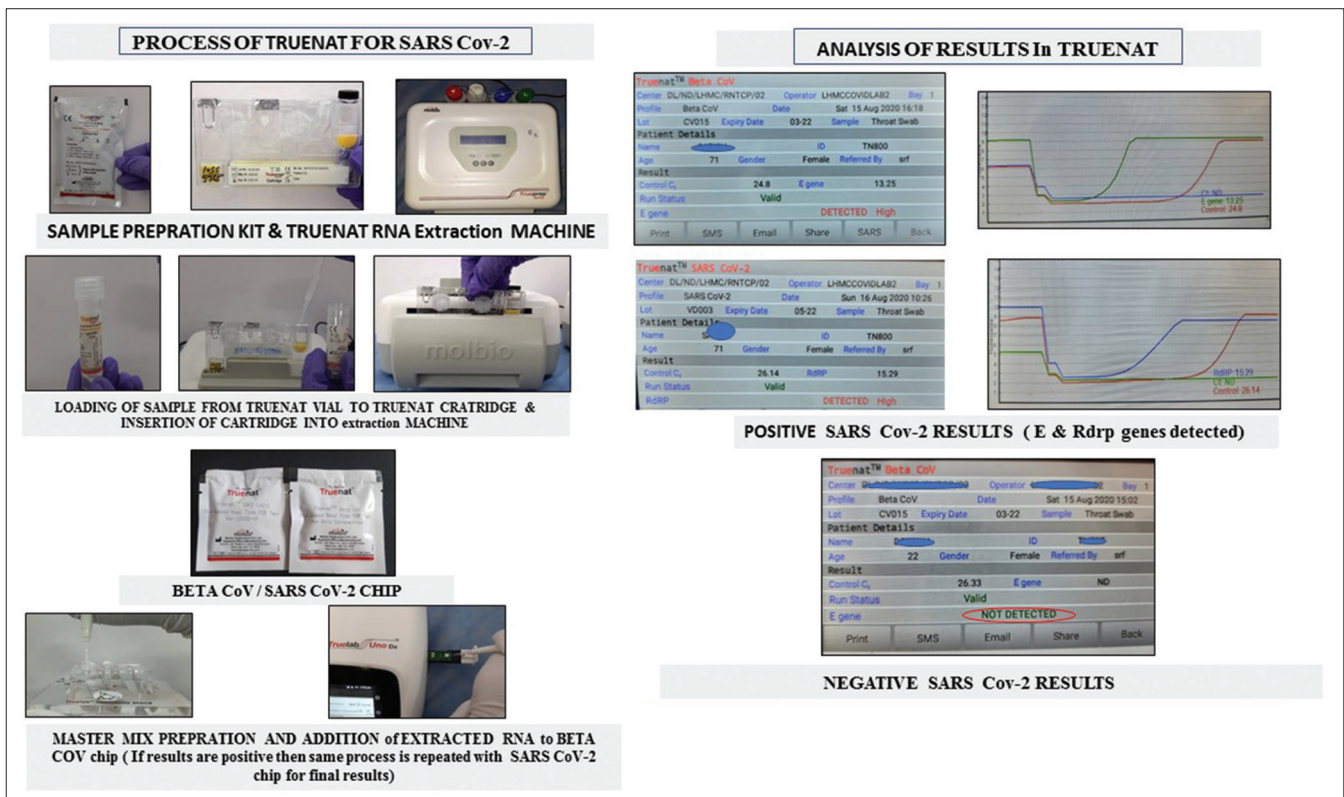


Figure 3: Workflow with analysis of results in TRUENAT platform for detection of SARS Cov-2

- perform the tests
- 2. Less technical expertise needed compared to conventional PCR
- 3. The results are easy to interpret as the results are available on the screen as detected/not detected./invalid
- 4. Less time consuming than PCR for setting up the test and time taken for the complete process of detection is also less compared to PCR.
- 5. A single sample can be put without wasting reagents. Helpful for emergency cases.
- 6. Machine is small (tabletop) and doesn't require much space in the laboratory.

Disadvantages of Truenat testing:

- 1. Only one sample can be processed by one Truenat machine at a time. So not an ideal instrument for a high throughput laboratory
- 2. Specialized vial is required to transport the sample
- 3. The sample in Truenat VTM can't be used for conventional RT PCR or CBNAAT
- 4. Doesn't show you any graph at the end of the test. So there is no chance of analysis of the characteristics of the graph and the corresponding result
- 5. In rare circumstances, mutations occurring within the highly conserved areas of the target genome where the Truenat assay primers and probes bind may result in false negative results.

Discussion

All the three amplification based methods for SARS-COV-2

diagnosis has its own pros and cons. Basic salient features of all the three methods are summarised in Table 2.

Choosing the right test for the right patient:

In choosing a diagnostic test for SARS-CoV-2, the most crucial point that differentiates these three methods is the time taken to perform the tests. In this pandemic time clinicians require the test results as soon as possible to isolate the patients and start required therapy.

The patients can be categorized as per their symptoms, comorbid conditions, demographic location and various other factors as mentioned below and any one of the three molecular based tools can be used for detection of SARS-CoV-2.

Category of patients for RT PCR testing of SARS-COV-2:

- 1. Influenza like illness cases with contact history with positive patients
- 2. Low risk symptomatic contacts of positive persons
- 3. Any adult patients with influenza like illness suspected of SARS-CoV-2 without co-morbidities with mild symptoms not requiring immediate intervention
- 4. Healthcare workers which require testing with high to medium risk of exposure
- 5. Symptomatic healthcare workers & other frontline workers
- 6. Patients coming from containment zone requiring admission in the hospital

Category of patients for CBNAAT & Truenat testing of SARS-COV-2:

- 1. All Severe acute respiratory illness (SARI) patients requiring

Table 2: Summarises the salient features of all the three methods

	RT PCR	CBNAAT	TRUENAT
Principle	Nucleic acid amplification : Real time	Cartridge based nucleic acid amplification	Nucleic acid amplification
Target genes	Varies with kits (mostly any combination of following genes in singleplex or multiplex form : E, N, ORF, S, Rdrp)	E & N gene Along with sample processing control	E & Rdrp
Biosafety requirements	Required (Minimum BSL 2 facility)	Required (Minimum BSL 2 facility)	Minimal
Sample transport	In Normal VTM	In Normal VTM	Special VTM with lysis buffer designed only for Truenat
Time taken (each run)	4-5 hrs for one run (additional extraction time of few hours: less time with automated extractor and more with manual method)	1 hr approximate	1 hr approximate including extraction time
No. of samples that can be processed in each run	Maximum 72, 96,384 samples (including controls) But most commonly 96 well real time PCR machine is used (Depends on the capacity of the Real time PCR machine)	Depends on the type and number of slots present in the machine. Maximum size 12 slots to process 12 samples at a time	One sample each time in one machine
Technical expertise	Good hands on Experience Required	Minimal (need basic training)	Minimal (need basic training)
Test procedure	Complex and involve multiple pipetting steps	Simple with minimal pipetting steps	Simple with minimum pipetting steps
Consumables & machines requirements	Relatively more compared to CBNAAT & TRUENAT due to multiple steps in the procedure	Less compared to RT-PCR	Less compared to RT-PCR

All the three methods are confirmatory test and there is no need to reconfirm or retest the sample by Real time PCR if the results are negative or positive by Truenat or CBNAAT

- immediate intervention
2. Dead bodies
 3. Paediatric patients with SARI waiting to be transferred to intensive care
 4. Before emergency surgical procedures wherever testing of SARS Cov is indicated
 5. Before delivery of pregnant lady coming from containment zone.

Key points to be noted while categorizing the patients for SARS-COV-2 testing:

It is important to remember that the above patient selection criteria for each test is arbitrary and might vary from institutions to institution and depends on the sample load of a particular testing facility. Moreover, as the number of samples that can be tested at a time in CBNAAT and truenat varies depending on the number and type of the machines available, every institution should note this point before deciding the turnaround time (TAT) and selection of patient categories for each testing modalities.

Summary and Conclusion

Considering the highly infectious nature and unexplained respiratory as well as multiorgan crisis related to COVID -19 disease, it is of utmost importance to maximize the testing capacity in order to isolate the patients and provide required treatment. Amplification based technologies as described above are fulfilling this need and the number of tests being conducted for SARS-CoV-2 are increasing day by day. Most of the laboratories in India are performing RT PCR using various kits as approved by ICMR and it is the constant endeavor of government to boost their testing capacity by adding CBNAAT & Truenat machines in various laboratories across India. RT PCR is a time tested technique and is considered to be the gold standard with good sensitivity & specificity for laboratory diagnosis of SARS-CoV-2 infection. A testing laboratory might take few hours depending on its sample load, man power and infrastructural availability to give a result of RT PCR based test for SARS-CoV-2. Moreover, it requires some stringent conditions and expertise starting from sample collection to sample processing and reading of results. Whereas, CBNAAT and Truenat testing Technologies demand less technical expertise as well as minimal technical and infrastructural requirements. The sensitivity and specificity of these testing modalities are also being validated and they are comparable to RT PCR. Like various other laboratory tools for diagnosis of infectious disease, these three diagnostic platforms for SARS-CoV-2 detection has its own limitations, that's why the treating physicians should understand the advantages and limitations of each method for SARS-CoV-2 testing so that they can use these diagnostic tools maximally & judiciously as per their requirement and availability in the interest of patient care.

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Conflicts of interest

There are no conflicts of interest.

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